APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Aug. 1996, p. 2845–2849 Vol. 62, No. 8
0099-2240/96/$04.00+0

Aminopeptidase N Purified from Gypsy Moth Brush Border Membrane Vesicles Is a Specific Receptor for *Bacillus thuringiensis* CryIAc Toxin

MI KYONG LEE,[1] TAEK H. YOU,[2] BRIAN A. YOUNG,[3] JEFFREY A. COTRILL,[1] ALGIMANTAS P. VALAITIS,[4] AND DONALD H. DEAN[1,2]*

Department of Biochemistry[1] and Department of Entomology,[2] The Ohio State University, Columbus, Ohio 43210; Molecular, Cellular, Developmental Biology and Genetics Program, University of Minnesota, St. Paul, Minnesota 55308[3]; and Northeastern Forest Experiment Station, USDA Forest Service, Delaware, Ohio 43015[4]

Received 26 April 1996/Accepted 29 May 1996

We have evaluated the binding of *Bacillus thuringiensis* Cry toxins to aminopeptidase N (APN) purified from *Lymantria dispar* (gypsy moth) brush border membrane vesicle (BBMV). CryIAc toxin bound strongly to APN, while either the structurally related CryIAa and CryIAb toxins or CryIC, CryIIA, and CryIIIA toxins showed weak binding to APN. An in vitro competition binding study demonstrated that the binding of CryIAc to *L. dispar* BBMV was inhibited by APN. Inhibition of short circuit current for CryIAc, measured by voltage clamping of whole *L. dispar* midgut, was substantially reduced by addition of phosphatidylinositol-specific phospholipase C, which is known to release APN from the midgut membrane. In contrast, addition of phosphatidylinositol-specific phospholipase C had only a marginal effect on the inhibition of short circuit current for CryIAa. These data suggest that APN is the major functional receptor for CryIAc in *L. dispar* BBMV. A ligand blotting experiment demonstrated that CryIAc recognized a 120-kDa peptide (APN), while CryIAa and CryIAb recognized a 210-kDa molecule in *L. dispar* BBMV. In contrast, CryIAa and CryIAb bound to both the 120- and 210-kDa molecules in *Manduca sexta* BBMV, while CryIAc recognized only the 120-kDa peptide. The 120-kDa peptide (APN) in *L. dispar* BBMV reacted with soybean agglutinin, indicating that *N*-acetylgalactosamine is a component of this glycoprotein.

The CryI family of insecticidal crystal proteins from *Bacillus thuringiensis* are toxic to lepidopteran larvae (26). A key step in the mechanism of action of *B. thuringiensis* toxins is the binding of toxin to specific receptors on the brush border membrane of midgut epithelium. In most cases, a positive correlation between toxicity and receptor binding to the specific receptor has been observed, although several exceptions are known (2a, 5, 6, 11, 24, 25, 28). After binding to the receptor, the toxin may be inserted into the midgut membrane, opening or forming cation-selective channels or pores leading to cell lysis and eventually the death of the insect (3). Currently, the insertion rate of CryIA toxins into the midgut membrane has been found to be the key factor for differences in their toxicity to *Lymantria dispar* (13).

In earlier studies, putative toxin binding proteins (receptors) in different insect brush border membrane vesicles (BBMVs) were identified and partially characterized by ligand blotting experiments (2a, 17, 18). CryIAb and CryIAc binding proteins from *Manduca sexta* have been identified as a 210-kDa protein (cahedrin-like protein) and a 120-kDa protein (aminopeptidase N [APN]), respectively, and their genes have been cloned (7, 19, 21, 22). In a previous study, we have purified APN from *L. dispar* BBMV and identified it as a CryIAc-specific binding protein (23).

It has been demonstrated that binding of CryIAc toxin to *M. sexta* APN is inhibited by *N*-acetylgalactosamine (GalNAc) and that soybean agglutinin (SBA) recognizes the APN in *M. sexta* BBMV (2a, 19). These data clearly indicate that GalNAc is an essential component of APN for interaction with CryIAc toxin. A recent study reported that the 120-kDa *M. sexta* APN is anchored in the membrane by a glycosylphosphatidylinositol (GPI) anchor and is converted to a 115-kDa soluble form by phosphatidylinositol-specific phospholipase C (PIPLC) (1). An endogenous PIPLC was reported to cleave the lipid moiety from the GPI anchor and convert 120-kDa APN to a free 115-kDa APN (14).

In the present study, we investigated the binding properties of *L. dispar* APN to different *B. thuringiensis* toxins. The effect of PIPLC on the activity of CryIAa and CryIAc was examined by voltage clamping assays with isolated *L. dispar* midguts. These data demonstrate that *L. dispar* APN is the major functional receptor for CryIAc toxin. BBMV ligand blotting assays probed with ^{125}I-labeled CryIA toxins and lectin (SBA) indicate that the 120-kDa CryIAc toxin-binding protein, APN, has a GalNAc component.

MATERIALS AND METHODS

Purification of the brush border membrane APN. BBMVs were prepared by the procedure of Wolfersberger et al. (27). The brush border membrane APN was purified from frozen *L. dispar* BBMV as described previously (23). Briefly, the 3-[(3-cholamidopropyl) dimethylamino]-1-propanesulfonate (CHAPS)-solubilized BBMV proteins were applied to an HR 5/10 Mono-Q column. Fractions containing aminopeptidase activity were pooled and concentrated. The enzyme was further purified by gel filtration on an HR 10/30 Superdex-75 column.

Purification of recombinant *B. thuringiensis* toxins. Crystal proteins were purified by the procedure described previously (11). Purified crystal proteins were solubilized in 50 mM sodium carbonate buffer (pH 9.5) containing 10 mM dithiothreitol at 37°C for 4 h. The solubilized protoxins were digested with 3% (wt/wt) trypsin at 37°C for 4 h. Activated toxins were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE [10% polyacrylamide]) (8).

Biotinylation and iodination of toxins. The trypsin-activated CryIAa, CryIAb, and CryIAc toxins were equilibrated in 0.1 M borate buffer (pH 8.0) and biotinylated according to the manufacturer's instructions (Boehringer Mannheim).

* Corresponding author. Mailing address: Department of Biochemistry, The Ohio State University, 484 West 12th Ave., Columbus, OH 43210. Phone: (614) 292-1738. Fax: (614) 292-6773. Electronic mail address: dean.10@osu.edu.

Biotinyl-*N*-hydrosuccinimide ester was dissolved in *N*,*N*-dimethylformamide (1 mg/ml) and added to each toxin sample with a 1:8 (wt/wt) biotin/toxin ratio. After incubation for 4 h at room temperature, the biotinylated toxins were separated from free biotin by dialysis or with an Amicon centricon. Iodination of toxins and APN was performed with IODOBEAD (Pierce) as described previously (11). Twenty-five micrograms of each toxin and 10 μg of APN were iodinated with 1 mCi of ^{125}NaI. Specific activity was measured by trichloroacetic acid precipitation of ^{125}I-labeled proteins. The specific activities of APN and the CryIAa, CryIAb, and CryIAc toxins were 1.8, 1.4, 1.3, and 1.5 μCi/μg, respectively.

Binding of ^{125}I-labeled APN to different *B. thuringiensis* toxins. Samples (2.5 μg) of CryIAa, CryIAb, and CryIAc protoxins and toxins and CryIC, CryIIA, and CryIIIA toxins were blotted onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad) with a slot blot apparatus (Bio-Rad). Membrane was blocked with 3% skim milk (Food Club) for 2 h and incubated with 4 nM ^{125}I-labeled APN for 2 h. The membrane was washed extensively with TTBS buffer (20 mM Tris, 500 mM NaCl [pH 7.5]) containing 0.05% Tween 20, dried, and exposed to Fuji-RX film.

Competition binding of CryIAc toxin between APN and BBMV. *L. dispar* BBMV was solubilized in 1% CHAPS–sodium carbonate buffer (pH 9.5) for 30 min on ice. Insoluble materials were removed by centrifugation at 13,500 × *g* for 15 min. Five micrograms of solubilized BBMV proteins was blotted onto PVDF membrane with a slot blot assay apparatus. Membrane was blocked with 3% skim milk for 2 h and incubated with 2.5 nM biotinylated CryIAc toxin in the absence and presence of increasing amounts of purified APN (1, 5, 10, 25, and 50 nM). Membrane was washed thoroughly with TTBS buffer, incubated with streptavidin-conjugated horseradish peroxidase (Boehringer Mannheim) for 1 h, and visualized with 3,3′-diaminobenzidine tetrahydrochloride (DAB) substrate (Sigma).

Voltage clamping analysis. Voltage clamping assays of short circuit current (Isc) were performed as described by Lee et al. (9). After the midgut from fifth instar *L. dispar* larvae had been stabilized for 20 min, 500 ng (2.2 nM) of CryIAa or CryIAc toxins was injected into the lumen side of the midgut. The inhibition of Isc was recorded with a Kipp and Zonen recorder, and data were analyzed with the MacLab data acquisition system. In order to examine the effect of PIPLC on the inhibition of Isc of toxins, 2 U of *Bacillus cereus* PIPLC (Sigma) was added to the lumen side of the chamber. After incubation for 30 min, 500 ng of CryIAa or CryIAc toxins was injected, and the inhibition of Isc was recorded by time.

BBMV ligand blotting. Twenty-five micrograms of *L. dispar* and *M. sexta* BBMV proteins was separated by SDS-PAGE (7.5% polyacrylamide) and transferred to PVDF membrane. After being blocked with 3% skim milk for 2 h, the membrane was incubated with 5 nM ^{125}I-labeled CryIAa, CryIAb, and CryIAc toxins for 5 h. The membrane was washed extensively with TTBS buffer, dried, and autoradiographed for 2 to 3 days. In order to examine the effect of detergent on toxin binding to the 120- and 210-kDa molecules in *L. dispar* BBMV, the BBMV proteins were transferred onto the membrane, and the membrane was treated with 0.5% Nonidet P-40 for 2 h at 37°C as described by Garczynski and Adang (2). The membrane was rinsed with TTBS and subjected to ligand blotting as described above. To identify the lectin binding proteins, 2.5 μg of BBMV proteins from *Heliothis virescens*, *L. dispar*, *M. sexta*, and *Bombyx mori* midguts was separated by SDS-PAGE (9% polyacrylamide), transferred to PVDF membrane, and incubated with biotin-labeled SBA (1 μg/ml) for 1 h. The membrane was probed with streptavidin-conjugated horseradish peroxidase (Boehringer Mannheim) and DAB substrate (Sigma).

The same set of BBMV proteins were separated by SDS-PAGE (7.5% polyacrylamide), transferred to PVDF membrane, and incubated with anti-APN antibody (1:3,000 dilution) for 1 h. The membrane was probed with goat anti-rabbit immunoglobulin G horseradish peroxidase conjugate (Bio-Rad) and DAB substrate (Sigma). For anti-APN preparation, about 250 μg of purified APN was subcutaneously injected into two New Zealand White rabbits. Rabbits were given booster injections 4 weeks later, and about 150 to 200 μg of antigen was injected. After 4 weeks, blood was collected via cardiac puncture, and about 150 ml of serum was collected.

RESULTS

Specific binding of CryIAc toxin to APN. The binding of different *B. thuringiensis* toxins to purified *L. dispar* APN was examined. Purified *B. thuringiensis* toxins were blotted onto PVDF membrane and probed with ^{125}I-labeled APN. Only CryIAc toxin exhibited strong binding to APN (Fig. 1, B3), whereas weak binding of CryIAa, CryIAb, CryIIA, CryIC, and coleopteran-specific toxin CryIIIA was observed only after a long exposure. Among the protoxins tested, only CryIAc protoxin exhibited weak binding to APN (Fig. 1).

CHAPS-solubilized BBMV was blotted onto PVDF membrane and incubated with biotin-labeled CryIAc toxin in the presence of increasing amounts of APN. The binding of CryIAc toxin to *L. dispar* BBMV was inhibited in proportion to the APN concentration (Fig. 2). However, inhibition was not complete even at a high concentration of APN.

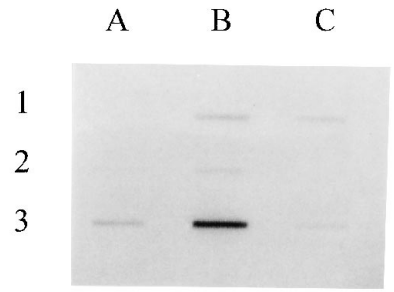

FIG. 1. Binding of ^{125}I-labeled APN to *B. thuringiensis* toxins. A total of 2.5 μg (each) of CryIAa (A1), CryIAb (A2), and CryIAc protoxins (A3) and CryIAa (B1), CryIAb (B2), CryIAc (B3), CryIC (C1), CryIIA (C2), and CryIIIA toxins (C3) was blotted onto PVDF membrane with a slot blot apparatus and probed with ^{125}I-labeled APN (4 nM). The membrane was washed, dried, and autoradiographed.

Inhibition of CryIAc activity by PIPLC measured by voltage clamping. We measured inhibition of Isc in response to the addition of toxins to the lumen side of *L. dispar* midgut. Isc measures the active transport of ions from the hemolymph side of midgut membrane to the lumen side (4). PIPLC is known to release aminopeptidase activity from *B. mori* and *M. sexta* midgut brush border membrane (1, 7, 20). The effect of PIPLC on the pore forming activity of CryIAa and CryIAc toxins was examined by voltage clamping. Two units of PIPLC was added to the lumen side of the midgut membrane, which was then incubated for 30 min. Next, 500 ng of CryIAa or CryIAc toxin was added, and the inhibition of Isc was examined. Complete inhibition of Isc was observed 30 min after addition of CryIAc in the nontreated midgut membrane, while 70% of the Isc remained in the PIPLC-treated midgut membrane. Sixty minutes after toxin addition, 50% of the Isc remained (Fig. 3A). CryIAa toxin completely inhibited Isc 30 min after toxin addition in the nontreated midgut membrane, while 30% of the Isc remained in the PIPLC-treated midgut membrane. Inhibition of Isc was complete 45 min after toxin addition (Fig. 3B).

Identification of toxin-binding proteins and lectin-binding proteins. BBMV ligand blotting experiments showed that CryIAa and CryIAb toxins bound to a 210-kDa *L. dispar* BBMV molecule, while CryIAc toxin recognized a 120-kDa peptide (Fig. 4A1). In contrast, in *M. sexta* BBMV, CryIAa recognized both 120- and 210-kDa molecules strongly. CryIAb exhibited strong binding to the 120-kDa molecule and weak binding to the 210-kDa molecule. CryIAc bound to only the 120-kDa peptide (Fig. 4B). The effect of the nonionic detergent Nonidet P-40 on binding of the CryIAa, CryIAb, and

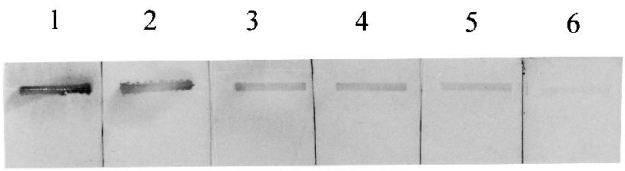

FIG. 2. Competitive binding of biotin-labeled CryIAc toxin between purified APN and BBMV. Five micrograms of CHAPS-solubilized *L. dispar* BBMV was applied to PVDF membrane and incubated with 2.5 nM biotin-labeled CryIAc toxin in the presence of increasing amounts of APN (lane 1, no APN; lane 2, 1 nM; lane 3, 5 nM; lane 4, 10 nM; lane 5, 25 nM; lane 6, 50 nM). The binding of biotin-labeled CryIAc toxin to BBMV was visualized with streptavidin-conjugated horseradish peroxidase and DAB substrate.

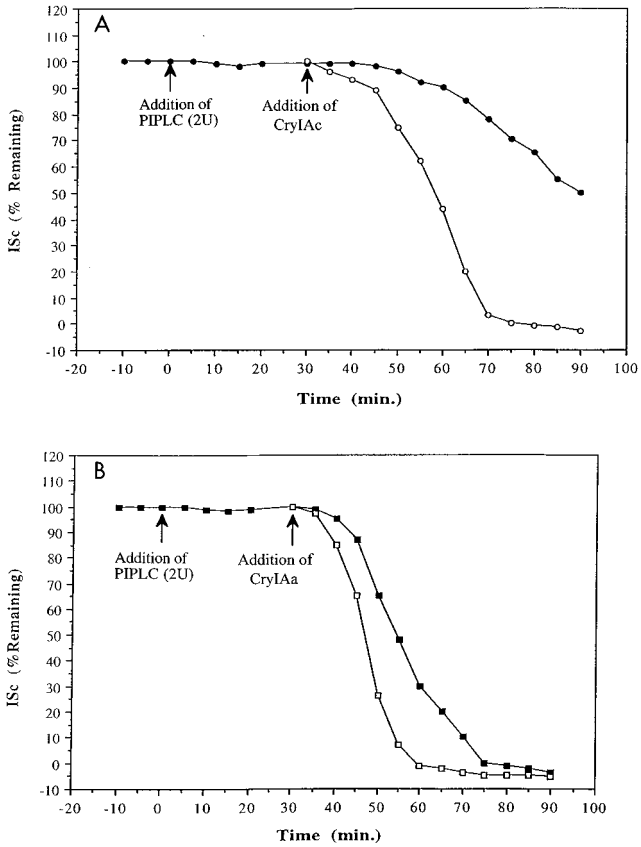

FIG. 3. Inhibition of Isc across *L. dispar* midgut. (A) Five hundred nanograms of CryIAc toxin (○) was injected into the lumen side of the chamber, and the drop in Isc was measured by time. To measure the effect of PIPLC, 2 U of PIPLC was added to the lumen side of the midgut and incubated for 30 min, and then 500 ng of CryIAc toxin (●) was added. The drop in Isc was measured by time. (B) Five hundred nanograms (2.2 nM) of CryIAa toxin (□) was injected, and the effect of PIPLC on the inhibition of Isc by CryIAa toxin (■) was examined.

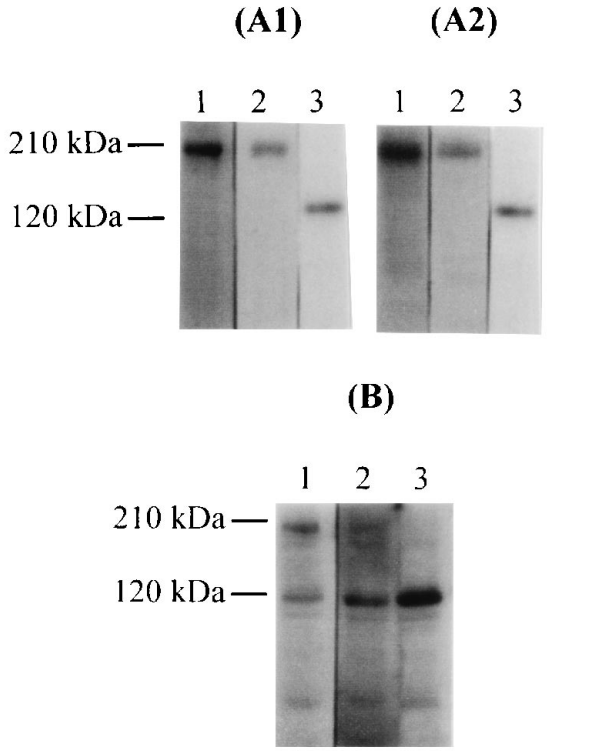

FIG. 4. Identification of CryIA toxin-binding proteins in *L. dispar* and *M. sexta* BBMV. (A) Twenty-five micrograms of *L. dispar* BBMV proteins was separated by SDS-PAGE (7.5% polyacrylamide) and transferred to PVDF membrane. The membrane was incubated with either TTBS buffer (A1) or TTBS with 0.5% Nonidet P-40 (A2) for 2 h. (B) Twenty-five micrograms of *M. sexta* BBMV proteins was transferred to the membrane and incubated with only TTBS buffer. A total of 5 nM (each) ^{125}I-labeled CryIAa (lane 1), CryIAb (lane 2), and CryIAc (lane 3) was used as a probe.

CryIAc toxins to *L. dispar* BBMV was examined. The binding properties of these toxins with the 210- and 120-kDa molecules were not altered by treatment with detergent (Fig. 4A2).

In order to identify lectin-binding proteins, BBMV proteins from different insects were transferred to PVDF membrane and probed with biotin-labeled lectin. We observed strong binding of the GalNAc-specific lectin SBA to the 170- and 120-kDa peptides in *Heliothis virescens* and the 120-kDa peptide in *L. dispar* and *M. sexta*. In *B. mori*, SBA bound to a peptide with a molecular mass slightly lower than 120 kDa. SBA did not bind to the 210-kDa molecule in either *M. sexta* or *L. dispar* BBMVs (Fig. 5).

Cross-reactivity of *L. dispar* APN antibody with different insect BBMV proteins. Antibody raised against *L. dispar* APN also reacted with BBMV proteins from different insects but to different degrees. Anti-APN antibody reacted strongly with the 120-kDa peptide (APN) in *L. dispar* BBMV. However, anti-APN antibody showed a weak interaction with the 120-kDa peptide in *M. sexta* and *H. virescens* and a slightly lower-molecular-mass peptide in *B. mori* (Fig. 6).

DISCUSSION

Recently, a receptor for *B. thuringiensis* CryIAc toxin has been purified from *M. sexta* BBMV and characterized. It has been demonstrated that CryIAc receptor is 120-kDa APN (2a, 7, 19). Previously, we have purified APN from *L. dispar* BBMV and identified it as a specific CryIAc-binding protein (23). In the present study, we further investigated the binding properties of APN with different *B. thuringiensis* toxins. Purified *B. thuringiensis* toxins blotted onto PVDF membrane were probed with ^{125}I-labeled APN. Strong binding of APN was observed only with CryIAc toxin. Structurally related CryIAa and CryIAb toxins or other *B. thuringiensis* toxins (CryIIA,

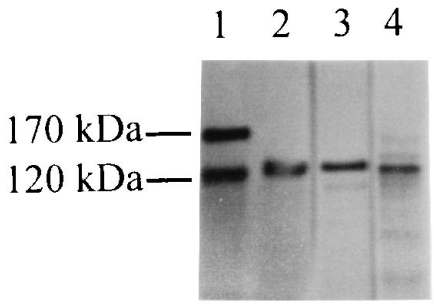

FIG. 5. Identification of lectin-binding proteins in different insect BBMVs. A total of 2.5 μg (each) of *H. virescens* (lane 1), *L. dispar* (lane 2), *M. sexta* (lane 3), and *B. mori* (lane 4) BBMV proteins was separated by SDS-PAGE (9% polyacrylamide) and transferred to PVDF membrane. The membrane was incubated with biotin-labeled SBA (1 μg/ml) for 1 h and probed with streptavidin-conjugated horseradish peroxidase and DAB substrate as described in Materials and Methods.

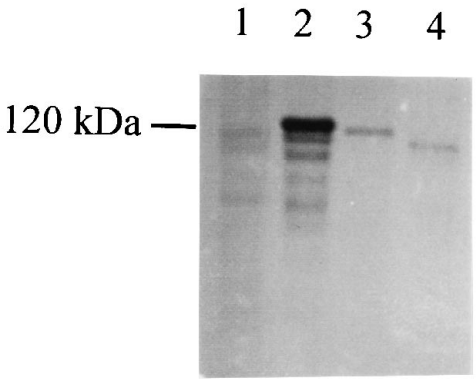

FIG. 6. Cross-reactivity of different insect BBMV proteins with *L. dispar* APN antibody. A total of 2.5 μg (each) of *H. virescens* (lane 1), *L. dispar* (lane 2), *M. sexta* (lane 3), and *B. mori* (lane 4) BBMV proteins was separated by SDS-PAGE (7.5% polyacrylamide) and transferred to PVDF membrane. Membrane was incubated with APN antibody and visualized with goat anti-rabbit immunoglobulin G and DAB substrate as described in Materials and Methods.

CryIC, and CryIIIA) showed weak binding to APN only after a long exposure (Fig. 1). Other Cry toxins, i.e., CryIIB, CryID, CryIF, and CryIVD, did not show significant binding to APN (data not shown). These data clearly demonstrate that APN is the specific binding protein for CryIAc toxin. In contrast, a recent kinetic binding study with surface plasmon resonance with APN purified from *M. sexta* demonstrated that CryIAa, CryIAb, and CryIAc toxins all bind to APN specifically (16). These data suggest the possibility of differences in the APN from these two insects, which might lead to differences in *B. thuringiensis* toxin-receptor binding. Also, the use of two different binding assay systems might lead to different conclusions.

Since we confirmed that APN is a specific CryIAc-binding protein, we have further examined whether binding of CryIAc toxin to BBMV can be inhibited by competition with APN. Binding of CryIAc toxin to *L. dispar* BBMV was inhibited in the presence of added APN, confirming that APN is the major CryIAc receptor in BBMV proteins (Fig. 2). However, inhibition was not complete even at high concentrations of APN. In another experiment, a fixed amount of APN was applied to PVDF membrane and incubated with biotin-labeled CryIAc toxin in the presence of increasing amounts of BBMV. The data also showed inhibition of binding of CryIAc toxin to APN (data not shown). Incomplete inhibition of binding of CryIAc to BBMV by APN might be due to the lower binding affinity of toxin to free APN than to APN within the BBMV. This possibility was tested by comparison of the binding affinities of toxin to APN and to BBMV.

Our previous in vitro competition binding assays with CryIAc and *L. dispar* BBMV demonstrated that the binding affinity and binding site concentration were 1.61 nM and 2.64 pmol/mg of BBMV proteins, respectively (10). Binding affinity, calculated from the binding assay, between CryIAc toxin and purified APN was about 100 times lower than that from the BBMV competition assay (10a). It was previously reported that the affinity of binding of CryIAc to *M. sexta* APN in phospholipid vesicles was 100 to 200 nM, which is about 100-fold lower than the affinity measured in binding assays with BBMV (19). The authors suggest the possibility that other membrane proteins or lipid components of the midgut membrane might contribute to higher binding affinity.

Although only a 120-kDa peptide (APN) was identified as a CryIAc toxin-binding protein in BBMV ligand blotting experiments (Fig. 4), we cannot preclude the possibility that CryIAc might recognize other receptors, which might not be stable under the denaturing conditions, so that we could not observe them in BBMV ligand blotting. Another possibility is that other membrane proteins, which might be functionally associated with the APN but not with toxin, might contribute to high-affinity binding of toxin.

It has been demonstrated that PIPLC releases aminopeptidase activity from *B. mori* midgut brush border membrane (20). APN located on the *M. sexta* midgut brush border membrane was reported to be anchored in the membrane by a GPI anchor and was released by *B. cereus* PIPLC (1, 17). PIPLC converts the amphipathic form of 120-kDa APN to a 115-kDa hydrophilic form. The 115-kDa APN also binds to CryIAc toxin (1, 14).

In this study, we investigated the effect of PIPLC on pore forming activity of CryIAc with *L. dispar* by voltage clamping assays. The inhibition of Isc by CryIAc after addition of PIPLC was significantly slower than inhibition by CryIAc alone (Fig. 3A). In contrast, PIPLC treatment had only a slight effect on the inhibition of Isc by CryIAa (Fig. 3B). These voltage clamping assay data clearly demonstrated that a GPI-anchored protein, possibly APN, is a major functional receptor for CryIAc and suggest that CryIAa recognizes a different receptor. PIPLC did not completely block the activity of CryIAc toxin. About 50% of the inhibition of Isc remained 60 min after addition of toxin (Fig. 3A). The incomplete blockage might be due to the partial release of APN by PIPLC, as reported by Garczynski and Adang (1). They observed that about 46% of the APN was released from *M. sexta* BBMV after exhaustive PIPLC digestion. Also, in *B. mori*, 40% of the aminopeptidase activity is released from midgut membranes by PIPLC (20). The slight effect of PIPLC on inhibition of Isc by CryIAa might be due to weak binding of CryIAa to APN, as seen in Fig. 1, although APN would not be the major receptor for CryIAa. Another possibility is that the 210-kDa CryIAa receptor also can be partially released by addition of PIPLC.

Previously, inconsistent observations were reported for CryIAb- and CryIAc-binding proteins with ligand blotting assays (2a, 15, 21). A recent study demonstrated that a 120-kDa APN from *M. sexta* was a common receptor for the CryIAa, CryIAb, and CryIAc toxins (16). In contrast, a CryIAb receptor has been purified and identified as a 210-kDa cahedrin-like protein, and its gene has been cloned (21, 22). Martinez-Ramirez et al. (15) have claimed that the 210-kDa protein is the common receptor for the CryIAa, CryIAb, and CryIAc toxins. Our ligand blotting experiments with *M. sexta* BBMV showed that CryIAa bound to both the 210- and 120-kDa molecules strongly. CryIAb showed strong binding to the 120-kDa molecule and weak binding to the 210-kDa molecule. CryIAc recognized only the 120-kDa peptide (Fig. 4B). Therefore, the identification of CryIA toxin-binding proteins in *M. sexta* BBMV remains controversial. One explanation for the inconsistency might be differences in the detection system, perhaps from the use of different detergents in washing the protein blots. In our ligand blotting assay procedure, Triton X-100 and Tween 20 were added at each step. In contrast to the case with *M. sexta*, in *L. dispar* BBMV, CryIAa and CryIAb bound only to the 210-kDa molecule, while CryIAc bound to the 120-kDa peptide. To investigate the effect of the detergent on the binding, PVDF membranes were treated with a nonionic detergent, Nonidet P-40, before being incubated with toxins. The binding of toxins to the 120- and 210-kDa molecules in *L. dispar* BBMV was not altered by treatment with Nonidet P-40 (Fig. 4A). Our ligand blotting data are in good agreement with the observations shown in Fig. 1. Examination

of the binding of different toxins to *L. dispar* APN clearly showed that the 120-kDa APN is the major specific receptor only for CryIAc (Fig. 1).

The CryIAc receptor, APN, in *M. sexta* has been reported to be a glycoprotein (7, 19). In the present report, we have examined whether the CryIAc-binding proteins in *L. dispar* and other insect BBMVs also have the glycoprotein properties. Strong binding of the GalNAc-specific lectin SBA to the 120-kDa peptide was observed in *L. dispar* and *M. sexta*. In *B. mori*, SBA bound to a peptide with a molecular mass slightly smaller than 120 kDa. These data indicate the presence of *N*-acetylgalactosamine in the 120-kDa CryIAc-binding protein. SBA did not bind to the 210-kDa molecule in either *M. sexta* or *L. dispar* BBMVs. We have previously reported that CryIAc toxin recognizes 170-, 150-, and 120-kDa peptides in *H. virescens* BBMV (12). Strong binding of SBA to 170- and 120-kDa peptides in *H. virescens* was observed (Fig. 5).

We also examined whether *L. dispar* APN is antigenically identical to APN in other insect BBMVs. Antibody raised against *L. dispar* APN also reacted with BBMV proteins from different insects, but to different degrees. Anti-APN antibody reacted strongly with the 120-kDa peptide (APN) in *L. dispar* but reacted weakly with the 120-kDa APN in *M. sexta* (Fig. 6). This observation and the sequence comparison between *L. dispar* APN and *M. sexta* APN (23) also support the possibility that the CryIAc receptor in different insects might have different properties.

In this study, we further examined the specific binding of CryIAc toxin to its purified receptor, APN, and clearly demonstrated that APN is a major functional receptor protein for CryIAc and that it contains a GalNAc moiety. This CryIAc receptor is distinct from the 210-kDa receptor for the CryIAa and CryIAb toxins.

ACKNOWLEDGMENTS

This research was supported by a grant from the National Institutes of Health (RO1 AI 29092) to D.H.D.

We thank Dan Zeigler for critical reading of the manuscript and Francis Rajamohan for helping with the voltage clamping assays.

REFERENCES

1. **Garczynski, S. F., and M. J. Adang.** 1995. *Bacillus thuringiensis* CryIA(c) δ-endotoxin binding APN in the *Manduca sexta* midgut has a glycosyl-phosphatidylinositol anchor. Insect Biochem. Mol. Biol. **25:**409–415.
2. **Garczynski, S. F., and M. J. Adang.** 1995. *Bacillus thuringiensis* CryIAc receptors (abstract), p. 21. *In* Abstracts of the 28th Annual Meeting of the Society for Invertebrate Pathology 1995. Cornell University, Ithaca, N.Y.

2a. **Garczynski, S. F., J. W. Crim, and M. J. Adang.** 1991. Identification of putative insect brush border membrane-binding molecules specific to *Bacillus thuringiensis* δ-endotoxin by protein blot analysis. Appl. Environ. Microbiol. **57:**2816–2820.

3. **Gill, S. S., E. A. Cowles, and P. V. Pietrantonio.** 1992. The mode of action of *Bacillus thuringiensis* endotoxins. Annu. Rev. Entomol. **37:**615–636.
4. **Harvey, W. R., and M. G. Wolfersberger.** 1979. Mechanism of inhibition of active potassium transport in isolated midgut of *Manduca sexta* by *Bacillus thuringiensis* δ-endotoxin. J. Exp. Biol. **83:**293–304.
5. **Hofmann, C., P. Luthy, R. Hutter, and V. Pliska.** 1988. Binding of the delta-endotoxin from *Bacillus thuringiensis* to brush-border membrane vesicles of the cabbage butterfly (*Pieris brassica*). Eur. J. Biochem. **173:**85–91.
6. **Hofmann, C., H. Vanderbruggen, H. Hofte, J. Van Rie, S. Jansens, and H. Van Mellaert.** 1988. Specificity of *Bacillus thuringiensis* delta-endotoxins is correlated with the presence of high-affinity binding sites in the brush border membrane of target insect midguts. Proc. Natl. Acad. Sci. USA **85:**7844–7848.
7. **Knight, P. J. K., B. H. Knowles, and D. J. Ellar.** 1995. Molecular cloning of an insect aminopeptidase N that serves as a receptor for *Bacillus thuringiensis* CryIAc toxin. J. Biol. Chem. **270:**17765–17770.
8. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
9. **Lee, M. K., A. Curtiss, C. E. Alcantara, and D. H. Dean.** 1996. Synergistic effect of the *Bacillus thuringiensis* toxins CryIAa and CryIAc on the gypsy moth, *Lymantria dispar*. Appl. Environ. Microbiol. **62:**583–586.
10. **Lee, M. K., and D. H. Dean.** 1996. Inconsistencies in determining *Bacillus thuringiensis* toxin binding sites relationship by comparing competition assays with ligand blotting. Biochem. Biophys. Res. Commun. **220:**575–580.

10a. **Lee, M. K., and D. H. Dean.** Unpublished observations.

11. **Lee, M. K., R. E. Milne, A. Ge, and D. H. Dean.** 1992. Location of *Bombyx mori* receptor binding region of a *Bacillus thuringiensis* δ-endotoxin. J. Biol. Chem. **267:**3115–3121.
12. **Lee, M. K., F. Rajamohan, F. Gould, and D. H. Dean.** 1995. Resistance to *Bacillus thuringiensis* CryIA δ-endotoxins in a laboratory-selected *Heliothis virescens* strain is related to receptor alteration. Appl. Environ. Microbiol. **61:**3836–3842.
13. **Liang, Y., S. S. Patel, and D. H. Dean.** 1995. Irreversible binding kinetics of *Bacillus thuringiensis* CryIA δ-endotoxin to gypsy moth brush border membrane vesicles is directly correlated to toxicity. J. Biol. Chem. **270:**24719–24724.
14. **Lu, Y.-J., and M. J. Adang.** 1996. Conversion of *Bacillus thuringiensis* CryIAc binding aminopeptidase to a soluble form by endogenous phosphatidylinositol phospholipase C. Insect Biochem. Mol. Biol. **26:**33–40.
15. **Martinez-Ramirez, A. C., S. Gonzalez-Nebauer, B. Escriche, and M. D. Real.** 1994. Ligand blot identification of a *Manduca sexta* midgut binding protein specific to three *Bacillus thuringiensis* CryIA-type ICPs. Biochem. Biophys. Res. Commun. **201:**782–787.
16. **Masson, L., Y. J. Lu, A. Mazza, R. Brousseau, and M. J. Adang.** 1995. The CryIA(c) receptor purified from *Manduca sexta* displays multiple specificities. J. Biol. Chem. **270:**11887–11896.
17. **Oddou, P., H. Hartmann, and M. Geiser.** 1991. Identification and characterization of *Heliothis virescens* midgut membrane proteins binding *Bacillus thuringiensis* δ-endotoxins. Eur. J. Biochem. **202:**673–680.
18. **Oddou, P., H. Hartmann, F. Radecke, and M. Geiser.** 1993. Immunologically unrelated *Heliothis* sp. and *Spodoptera* sp. midgut membrane-proteins bind *Bacillus thuringiensis* CryIA(b) δ-endotoxin. Eur. J. Biochem. **212:**145–150.
19. **Sangadala, S., F. Walters, L. H. English, and M. J. Adang.** 1994. A mixture of *Manduca sexta* aminopeptidase and phosphatase enhances *Bacillus thuringiensis* insecticidal CryIAc toxin binding and ^{86}Rb-K efflux *in vitro*. J. Biol. Chem. **269:**10088–10092.
20. **Takesue, S., K. Yokota, S. Miyajima, R. Taguchi, H. Ikezawa, and Y. Takesue.** 1992. Partial release of APN from larval midgut cell membranes of the silk worm, *Bombyx mori*, by phosphatidylinositol-specific phospholipase C. Comp. Biochem. Physiol. **102B:**7–11.
21. **Vadlamudi, R. K., T. H. Ji, and L. A. Bulla, Jr.** 1993. A specific binding protein from *Manduca sexta* for the insecticidal toxin of *Bacillus thuringiensis* subsp. *berliner*. J. Biol. Chem. **268:**12334–12340.
22. **Vadlamudi, R. K., E. Weber, I. Ji, T. H. Ji, and L. A. Bulla, Jr.** 1995. Cloning and expression of a receptor for an insecticidal toxin of *Bacillus thuringiensis*. J. Biol. Chem. **270:**5490–5494.
23. **Valaitis, A. P., M. K. Lee, F. Rajamohan, and D. H. Dean.** 1995. Brush border membrane aminopeptidase-N in the midgut of the gypsy moth serves as the receptor for the CryIA(c) δ-endotoxin of *Bacillus thuringiensis*. Insect Biochem. Mol. Biol. **25:**1143–1151.
24. **Van Rie, J., S. Jansens, H. Hofte, D. Degheele, and H. Van Mellaert.** 1989. Specificity of *Bacillus thuringiensis* δ-endotoxins: importance of specific receptors on the brush border membrane of the midgut of target insects. Eur. J. Biochem. **186:**239–247.
25. **Van Rie, J., W. H. McGaughey, D. E. Johnson, B. D. Barnett, and H. Van Mellaert.** 1990. Mechanism of insect resistance to the microbial insecticide *Bacillus thuringiensis*. Science **247:**72–74.
26. **Whiteley, H. R., and H. E. Schnepf.** 1986. The molecular biology of parasporal crystal body formation in *Bacillus thuringiensis*. Annu. Rev. Microbiol. **40:**549–576.
27. **Wolfersberger, M., P. Luthy, A. Maurer, P. Parenti, V. F. Sacchi, B. Giordana, and G. M. Hanozet.** 1987. Preparation of brush border membrane vesicles (BBMV) from larval Lepidopteran midgut. Comp. Biochem. Physiol. **86A:**301–308.
28. **Wolfersberger, M. G.** 1990. The toxicity of two *Bacillus thuringiensis* δ-endotoxins to gypsy moth larvae is inversely related to the affinity of binding sites on midgut brush border membrane for the toxins. Experientia **46:**475–477.